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14. ABSTRACT

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15. SUBJECT TERMS

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Two-photon excited fluorescence from biological aerosol particles

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Abstract

We used a 40 MHz mode-locked 524 nm laser source to evaluate the utility of sub-picosecond excitation of fluorescence from 2-photon absorption in biological aerosols. Individual particles of biological composition, as well as other calibration particles, suspended in an inlet air flow were illuminated and measured as they passed through an optical chamber. To our knowledge, this is the first demonstration of 2-photon excited fluorescence from micron-sized particles composed of micro-organisms. We also observed a high fluorescence signal at visible wavelengths, which was not present with single-photon excitation.

Laser Induced Fluorescence; Particle Scattering; 2-photon excitation; aerosol; biological particle.

In addressing concerns about bio-terrorism and bio-warfare there has been significant activity in recent years toward development of new methods for sensing the presence of biological aerosols. Many of these newly-developed bio-threat detection systems have used laser-induced fluorescence (LIF) to provide an initial rapid indication of the presence of biological aerosol particles. Examples of recent ultraviolet (UV) LIF sensor systems include: BAWS [1], PFS [2] and SPFA [3, 4] as well as others [5]; and while their performance capabilities may vary, it is generally acknowledged that the basic approach offers rapid discrimination between fluorescent and non-fluorescent aerosol particles, and that LIF emission is highly correlated with biological composition [1-7]. This study was initiated to evaluate alternate excitation sources for probing bio-aerosols; in particular we explore the applicability of mode-locked laser to excite 2-photon absorption and subsequent fluorescence in bio-aerosols as a detection signature. Such laser sources might provide one avenue for developing sensors with reduced size, power and cost. To our knowledge the only other multi-photon work on aerosols has been conducted by Boutou et. al [8]. They had reported both theoretical and experimental angularly resolved multi-photon fluorescence from coumarin in ethanol droplets and tryptophan in nominally 50 μm water droplets.

Mode-locked fiber lasers are capable of generating short pulses with high peak powers and have the additional advantages of being potentially compact and rugged. Second harmonic generation (SHG) from these mode-locked lasers can produce light in the visible spectral region, while two sequential SHG stages will result in a UV source that can be used for a bio-sensor. Visible laser sources, however, have significantly longer lifespans compared to currently employed UV sources, primarily due to defects that develop over time in the non-linear crystals of the UV SHG stage. Since visible sources require only one SHG stage, they can be more

rugged, energy efficient, and more cost effective compared to similar lasers incorporating an additional SHG stage to reach the UV spectral region for excitation.

In terms of a fiber laser design, the short pulses produced by mode-locked lasers cannot be directly generated at the high powers necessary for efficient SHG because of detrimental nonlinear effects within the fiber medium that result in material damage. We overcame these limitations by building a band-limited Yb-doped fiber laser with no dispersion compensation [9], as the master oscillator for a chirped-pulse-amplification setup. With this design we have achieved greater than 220-kW peak power output pulses in the IR. The master oscillator was an all-normal-dispersion Yb-doped fiber laser [9], followed by high-dispersion fiber for stretching the pulses, a single-mode Yb-doped fiber amplifier, and finally a chirped volume Bragg grating to recompress the pulses. The recompressed pulse had an autocorrelation width of ~670 fs in duration, and its expected transform-limited pulse width for a normal-dispersion laser with this spectral width would be ~454 fs [10]. For these experiments, a SHG stage consisting of a 5 x 5 x 20 mm lithium borate crystal was used to frequency-double from a 1048 nm to a 524 nm wavelength. The full-width-half-maximum spectral width of the 524 nm pulses was ~0.8 nm. A fused silica prism was used to separate the 524 nm from the residual 1048 nm.

In order to study 2-photon excited fluorescence, an earlier aerosol interrogation apparatus originally developed for studying single UV photon excited fluorescence from particles was modified. While details of this basic system have been described elsewhere [11, 12], a short overview is provided here. As illustrated in Fig. 1, the aerosol interrogation apparatus consists of four main parts: a 785 nm CW laser module, 524 nm mode-locked fiber laser, an aerosol chamber, and a fluorescence and elastic scattering detection spectrometer.

A continuous wave (CW) laser diode at 785 nm was used to detect the presence of a particle in the focal volume via elastic scattering, which initiates all timing, interrogation and data collection processes, and provided a relative indication of particle size from the magnitude of scatter. The CW beam was split into two orthogonally polarized beams by using a birefringent crystal and these were focused using a cylindrical lens to form two flattened parallel beams in the focal volume of the aerosol chamber. A time-of-flight measurement between the two beams was used to calculate the velocity of each individual particle [11, 12] from which, the energy of the mode-locked beam incident on the particle could be calculated.

The sample air stream was introduced to the air-tight aerosol chamber through an inlet nozzle (700 μm diameter final aperture) mounted on top of the chamber, and the air flow continued in a straight line through the chamber and out a 6 mm diameter exit tube in the chamber bottom. The particles suspended in the air stream transit through the aerosol chamber interact with the laser beams at the focal point of the elliptical collection mirror in the ($\approx$ 1 cm) gap between the inlet nozzle and the exit tube. Scattered light and fluorescent emission from particles was collected by the elliptical mirror with a solid angle slightly greater than 2π steradians, and directed along the optical axis to a pair of collimating lenses. The collimated light from these lenses was transmitted to the fluorescence and scatter detection module.

The fluorescence and scatter detection module consisted of a series of photomultiplier tubes (PMTs) that were used to detect both elastically scattered light and fluorescence from individual aerosol particles. The basic design consisted of a series of custom beam splitters arranged linearly along the optical axis. Each of these dichroic beam splitters was fabricated to reflect/transmit specific wavelengths of interest onto each PMT as a specific spectral data channel (numbered in Fig. 1). Looking from left to right in Fig. 1, the first optic was a BG3 filter

that blocked most of the 524 nm scattered light and transmitted the scattered CW laser light at 785 nm and the fluorescence light from 300 nm to 470 nm. The second beam splitter (for PMT 1) was designed to reflect light only around 266 nm and this channel was not used during this study. The third beam splitter reflected light in a wavelength band between 300 nm and 400 nm (into PMT 2), but transmitted light longer than 400 nm. The fourth beam splitter in the linear chain along the main optical axis was a HR reflector at 532 nm that reflected the scattered light to be detected in PMT 5. The next beam splitter reflected light in a band between 400 nm and 470 nm (PMT 4) and passed longer wavelengths. Spectral (cutoff) filters were used in each of the channels to transmit only light within the desired band, and prevent detection of any stray scattered light. A condensing lens was also used in each of the channels to focus the collimated light onto the PMT light sensitive anodes. The last two PMTs (6 and 7) depicted in Fig. 2 are employed to detect elastically scattered light from the two orthogonally polarized 785 nm CW laser beams. Since one of these PMT channels has a polarizing filter in place, taking the ratio of these two channels permits assignment of which beam the particle scattering originates [11, 12].

The 524 nm mode-locked fiber laser was aligned to counter-propagate along the CW 785 nm laser beam. The 524 nm beam was sampled by a photodiode to provide an average power monitor on a particle-by-particle basis. The mode-locked laser was focused to form a 55 μm X 80 μm beam in the focal volume of the elliptical collection mirror. For particles flowing at a median velocity of roughly 4.4 m/s, this implied an average particle transit time of 18 μs, during which ~720 pulses would be incident on the particle. The maximum effective fluence of the 524 nm laser was measured as 463 mJ/cm^2. In order to capture the signal from all the laser pulses that are incident on a particle during its transit through the beam, the electronic gate width for the integrator was set to 100 μs.

The fluorescence response for several different sizes of commercially available PSL particles and lab-generated BW simulants using single photon excitation has been previously well characterized using systems similar to the one described here, and are discussed in detail elsewhere [4, 13]. For the observations reported here, 2-photon excited fluorescence was measured in two emission spectral channels centered at 350 (300nm to 400 nm) and 435 nm (400 nm to 470 nm) respectively, as well as the elastic scatter intensity at 524 nm. These measurements were made on PSL sphere aerosols (both pure and dye-doped) for several known sizes, as well as biological aerosol particles. PSL particle aerosols were generated using a collision nebulizer. The biological aerosol particles were produced using a Sono-Tek™ spray generator with water solutions/suspensions of the biological material. As the water evaporates from the droplet, it leaves a residual dry particle of the dissolved and/or suspended material. By controlling the concentration of the aerosol material in solution, the size of the resulting aerosol particles could be varied over a range from 1 to 7 μm in diameter.

The measured fluorescence in the 350 nm band is plotted in Fig. 2(a) as a function of the measured elastic scatter for a series of sizes of PSL spheres. The data is represented as scatter plot where each dot corresponds to the integrated signal intensity from an individual aerosol particle. The measured fluorescence signal has been corrected for various experimental parameters including the gain and spectral response of the PMT detector, the transmission of the various filters and optics, and has been converted to number of photons normalized to the square of the incident intensity. Consequently, the measured signal is represented as the number of photons emitted per J^2/cm^4 of incident light.

In our prior bio-aerosol work, the excitation beam was focused using a cylindrical lens to form a sheet beam of roughly 1.5 mm X 200 μm, such that the laser beam was wider than the

700 μm diameter aerosol stream [4, 11-13]. This enabled us to sample all the incoming particles with fairly uniform excitation intensity. However, for this work, the beam had to be much smaller than the particle stream in order to attain sufficient incident fluence. Since the data acquisition is initiated by the elastic scatter signal from the CW 785 nm laser beam which is much wider (4 mm) than the particle flow, all the particles traversing through the system are detected, but only a subset of these particles will actually be in the 524 nm excitation beam. Therefore the majority of particles that transit through the system register little or no signal from the 524 nm beam. For each particle type in this study, only 8-10% of the particles recorded are actually excited by the 524 nm beam. This effect can be seen in Fig. 2(a). For each particle size sampled, signal maxima are measured when the particles are located at the center of the excitation beam, but a continuous range of fluorescence values from this maximum down to the baseline (when the particles totally miss the beam) are recorded. By plotting a histogram of the emission values, an acceptance threshold could be selected, and particles transiting near the center of the beam can be separated for comparison and analysis. Fig. 2(b) shows the same data as Fig. 2(a) after this threshold procedure has been applied.

   Two-photon excited fluorescence measured for a series of particle sizes of Tryptophan DL (Sigma-Aldrich) is shown in Fig. 3. The fluorescence measured in the 350 nm band is plotted vs. the 435 nm band fluorescence. Three sizes of tryptophan particles were generated with the median diameters of: 1.5 μm, 3.8 μm and 5.4 μm, and distribution standard deviations of less than 40%. The fluorescent emission is seen to increase for increasing particle size. However, the measured fluorescence signal is broader compared to the signals measured for the PSL, due to the broader size distribution of the tryptophan particles. To quantify the 2-photon fluorescence response as a function of particle size, the signal measured for the tryptophan and

PSL series is plotted in Fig. 4 as a function of their mean aerodynamic particle size, measured

independently using an APS 3321 (TSI Inc.). The fluorescence intensities measured in the 350

nm band, 435 nm band and the elastic scatter at 524 nm are shown. The data shown in Fig. 4 has

been fitted to a power-law dependence that is plotted as a solid line and labeled for each of the

bands. The fluorescence centered in the 350 nm band scales roughly to the cube of the diameter

of the particle for both the PSL particles and the tryptophan particles. This relationship was also

observed previously using single-photon UV excitation [14]. For tryptophan particles the

fluorescence measured in the band centered at 435 nm shows a power-law dependent exponent

of 2.7, which is fairly in good agreement with an emission dependence proportional to particle

volume. For particles composed of a heterogeneous mixtures (e.g. agglomerates of spores in

media), this dependence was observed to deviate from the cubic dependence more significantly

[14]. As expected, the PSL particles do not emit much native fluorescence in the 435 nm band,

and the signal levels were too weak to establish particle size dependence. The elastic scatter

signal is roughly proportional to the size of the particle, its dependence on particle size will have

a more complicated form due to the fact that the angular dependence of the elastic scatter is a

strong function of the particle size. Nevertheless, fitting the elastic scatter signal to a power-law

dependence results in an exponent of 1.7 for PSL and 1.4 for tryptophan. Mie theory

calculations performed taking into account the geometry of the elliptical mirror yield a

theoretical dependence on particle size of 1.6 for the PSL particles using the known value of the

refractive index, and this result is consistent with the experimental value to within experimental

error.

    True two-photon excited fluorescence will have a quadratic dependence on the excitation

intensity. In order to verify this relationship the fluorescence signal was measured as the

incident excitation power was attenuated over a range from 900 mW to 200 mW. The 2- photon

excited fluorescence and elastic scatter was measured for 3.3 μm tryptophan and 2 μm dye-

doped PSL particles. The signal in the two fluorescence bands, centered at 350 and 435 nm, and

elastic scatter channel is plotted as a function of excitation power along with their power-law fit

in Fig. 5. In Fig. 5, the elastic scatter signals for both particle materials scale linearly with the

excitation energy, as one would expect. However, for both types of particles, the fluorescence

values in both the fluorescence bands show a dependence on the excitation energy a of 2 within

experimental error, validating that the emission results from a 2-photon absorption process.

The fluorescence signals measured for the bacterial samples of *Bacillus Globigii* (BG) and

Yersinia Rohdei (YR) are plotted together with the signature of a non-fluorescent sample

material, kaolin, in Fig. 6(a) and 6(b) respectively. The YR samples were washed in saline

(0.06%) while the BG samples contained spent sporulation media. The fluorescence band

centered at 350 nm is plotted versus the fluorescence band centered at 435 nm for particle size

distributions centered at 2.6 μm and 1.7 μm for YR, 3.8 μm and 2.5 μm for BG and 2.3 μm for

kaolin. Similar to ovalbumin particles discussed previously, these particles were also generated

using the Sono-Tek generator, and hence exhibited a broader particle size distribution than PSL

particles, but with a standard deviation of less than 40% of their mean diameter. Heterogeneous

composition, wider size distributions and lower SNR's for these weakly fluorescing samples all

contribute to the spread of data observed in Fig. 6. Nevertheless, fluorescence signals for the

biological particles demonstrate easily separable mean values relative to kaolin particle

signatures.

A 2-photon excited emission cross-section can be calculated from the measured fluorescence

signal [15]. For single particle detection where the particle is much smaller than the beam (i.e.,

the beam intensity doesn't vary much over the dimension of the particle) and for a Gaussian

beam profile the 2-photon fluorescence cross-section can be expressed as:

$$\sigma_{Fl} = \frac{Fl_{cor}}{\# \ pulses} \times \frac{1}{CV\tau} \times \frac{2}{.66} \times \frac{1}{I_p^{\ 2}} \qquad (1)$$

where Fl_{cor} is the fluorescence signal corrected for the instrument response, # pulses corresponds

to the number of pulses that was incident on the particle, C is the concentration, V is the volume

of the particle, τ is the pulse width and I_p is the peak intensity of the excitation source [15].

Using Eq. 1, emission cross-sections for different types of particles are listed in Table 1 as

calculated from the measured fluorescence signal. Various size particles were generated for each

type of particle material listed, and the 2-photon fluorescence signal was measured. Since the

absolute number of fluorophores present in each particle is unknown, the 2-photon cross-section

was computed on a per-particle basis, and a uniform, homogenous concentration of fluorophores

within the particle is assumed. Since fluorescence signals are expected to scale linearly with the

volume of the particle, the values of the cross-sections for each sample material have been

normalized to the cube of the particle diameter for the different sizes of particles. An average

was computed from the normalized cross-section obtained for the varying size particles and the

resulting cross-section values, nominally for one-micron spherical particles of each material, are

presented in Table. 1. The uncertainties in these measurements are estimated to be about a factor

of $\pm$ 2, due to the broad particle size distribution, the variation in the signal because of the spatial

distribution of the particles, and the Gaussian excitation beam being much smaller than the

particle flow.

particle type	median particle sizes (μm)	350 nm fluor CS (cm^4.S/photons /micron particle)	435 nm fluor CS (cm^4.S/photons /micron particle)
kaolin	2.4	2.1E-45	2.0E-46
BG	2.5, 3.8	2.8E-45	1.7E-45
YR	1.7, 2.6	4.7E-45	5.0E-45
ovalbumin	1.8, 3.3, 5.4	5.8E-45	5.6E-45
tryptophan	2.6, 3.8, 5.4	9.7E-45	2.2E-45
PSL	1, 2, 3, 5	8.5E-44	1.7E-45
PSL 450 dye doped	1 ,2	1.4E-43	2.0E-43
coumarin 450 in ethanol	3	1.1E-45	3.5E-44

Table. 1. The measured 2-photon fluorescence cross-section for a number of biological simulants, interferents and calibration particles.

Since tryptophan particles are homogenous in composition, and the concentration of tryptophan in the original droplet solution is known, the 2-photon fluorescence cross-section per molecule can also be calculated for these particles. In the 350 nm band the value is $4.7\pm1.4 \times10^{-54}$ cm^4.S/photon/molecule, and for the 435 nm band it is $1.1\pm1 \times10^{-54}$ cm^4.S/photon/molecule. A previously reported value for 2-photon absorption cross-section of tryptophan is 3.09×10^{-51} cm^4.S/photon/molecule at 532 nm [16]. Using the reported value of 0.21 for the quantum yield of tryptophan, the fluorescence cross section of 3.2×10^{-52} cm^4.S/photon/molecule can be calculated [16]. Our measured cross-section is a factor of 68 lower compared to previously published value. However, the cited work was performed in dilute aqueous solutions, while in our study the particles were formed from droplets in which the water is evaporated away, leaving a core of solid material with roughly 10^{10} molecules present in a 2.5 μm particle. Therefore the lower fluorescence cross sections observed in this work, likely includes quenching effects from neighboring molecules, re-absorption of the fluorescent emission, and non-uniform absorption of the incident excitation beam.

Looking at the cross-section data from Table. 1, an obvious feature is the relatively high fluorescence cross-section obtained in the 435 nm band compared to that of 350 nm emission band for all of the biological sample materials. In previous data from single-photon excitation, the relative cross-sections of the 435 nm band are typically two orders of magnitude lower than the 350 nm band for particles such as tryptophan and ovalbumin and a slightly smaller ratio for bacterial cells [4]. This is in sharp contrast to the observed 2-photon absorption data shown in Table 1 where the ratios of the emission intensity from the 350 nm band to the 435 nm band vary from approximately unity to four over the four different biological materials listed.

Blue-green fluorescence termed 'hyperluminscence' has been previously observed from serotonin (5-HT) and its precursor hyrdroxytryptophan (5-HTP) using multi-photon excitation [17-19]. Visible emission from tryptophan has also been observed from 3-photon excitation using Ti:sapphire laser at 795 nm [20]. Nishimura and Kinjo [20] also report observing blue-green fluorescence for incident power levels well below the breakdown value in pure water (peak power densities greater than $1.3X10^{13}$ W/cm^2). While the origin of this emission continues to be debated (see for example, Dad et al. [18] and Botchway et. al [21]), these same phenomena could be responsible for the emission that we see here in the 435 nm band for aerosol particles composed of proteins and bacterial particles only.   For the results reported here, the maximum peak power density incident on the particles was $1.3X10^9$ W/cm^2, well below the breakdown threshold for water. Further work will be required to understand and characterize this anomalously high emission in this spectral region.

In summary, we have shown dependence of particle emission proportional to the square of excitation intensity, proving its 2-photon excited nature, from individual particles of several biological compositions. We have demonstrated discrimination between biological aerosol

particles and kaolin particles based on 524 nm excited 2-photon fluorescence. To our knowledge these are the first observations of 2-photon excited fluorescence from micron-sized microbial particles. Additionally we have measured a linear dependence of fluorescent emission as a function of the particle volume for homogenous particles such as tryptophan in this size range. We also observed 2-photon excited fluorescence in the visible band from the bio-aerosols, which was not present from similar particles with single photon UV excitation.

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Figure Captions:

Figure 1.   Schematic layout of the Aerosol Interrogation arrangement and the 524 nm Mode-locked fiber laser.

Figure 2. 2-photon excited fluorescence of varying size Polystyrene Latex Spheres; showing measurement (a) from all particles transiting through the aerosol interrogation system (b) from only particles going through the center of the mode-locked laser.

Figure 3. 2-photon excited fluorescence from varying size individual Tryptophan particles.

Figure 4. 2-photon excited fluorescence and elastic scatter dependence on particle size for PSL and tryptophan particles demonstrating a volume dependence.

Figure 5. 2-photon excited fluorescence and elastic scatter dependence on excitation intensity demonstrating a quadratic and linear dependence respectively.

Figure 6. 2-photon excited fluorescence from biological simulants showing good separation between biological stimulants and inteferent material kaolin.